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SPECIAL ISSUE ARTICLE

The DAC system and associations with acute leukemias and myelodysplastic syndromes

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Summary Imbalances of histone acetyltransferase (HAT) and deacetylase activity (DAC) that result in deregulated gene expression are commonly observed in leukemias. These alterations provide the basis for novel therapeutic approaches that target the epigenetic mechanisms implicated in leukemogenesis. As the acetylation status of histones has been linked to transcriptional regulation of genes involved particularly in differentiation and apoptosis, DAC inhibitors (DACi) have attracted considerable attention for treatment of hematologic malignancies. DACi encompass a structurally diverse family of compounds that are being explored as single agents as well as in combination with chemotherapeutic drugs, small molecule inhibitors of signaling pathways and hypomethylating agents. While DACi have shown clear evidence of activity in acute myeloid leukemia, myelodysplastic syndromes and lymphoid malignancies, their precise role in treatment of these different entities remain to be elucidated. Successful development of these compounds as elements of novel targeted treatment strategies for leukemia will require that clinical studies be performed in conjunction with translational research including efforts to identify predictive biomarkers.

Keywords Epigenetic therapy · Deacetylase inhibitors · Myelodysplastic syndromes · Acute myeloid leukemia · Drug development · Targeted therapy · Hypomethylating agents

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Histone deacetylases as therapeutic targets in hematologic malignancies

Among the epigenetic modifications associated with the development of human cancer, alterations of the acetylation status of histones play a prominent role (see [1] for review). The extent of acetylation and deacetylation on different positions of core histones is determined by the antagonistic activity of histone acetylases (HAT) and histone deacetylases (HDAC) and alters the nucleosomal conformation of both transformed and non-transformed cells. Deacetylation of histones by HDACs hinders the accessibility of DNA to transcription factors that are involved in determining malignant cell behaviour, thereby changing their activity, subcellular localisation and interaction partners. In addition, acetylation is an important post-translational modulation of a wide range of nuclear and cytoplasmic proteins involved in the regulation of a multitude of cellular functions (e.g., p53, tubulin, heat-shock protein 90). A disrupted equilibrium between HDACs and HATs, with preponderance of deacetylase activity, leads to transcriptional repression of a diverse set of genes involved in the regulation of cell proliferation, differentiation and apoptosis. Aberrant gene transcription caused by abnormal activity of HATs and HDACs is commonly observed in leukemia and lymphoma [2–6]. Accordingly, modulation of protein lysine acetylation through inhibition of histone deacetylases (HDACs) is currently being considered as an attractive new therapeutic strategy for acute myeloid and lymphoid leukemias and myelodysplastic syndromes.

Involvement of HDACs in myeloid malignancies (AML and MDS)

Aberrant HDAC activity in acute leukemia

In acute myeloid and lymphoid leukemia, recurring chromosomal translocations result in the generation of chimeric fusion genes, many of which function as transcriptional regulators. Several of these fusion proteins are associated with the development of leukemia, partly by causing transcriptional deregulation of genes responsible for differentiation and inhibition of apoptosis through mechanisms linked to chromatin alterations. Binding of oncogenic transcription factors such as PML-RARα, PLZF-RARα, AML1-ETO, and others, induce deposition of aberrant chromatin marks by recruiting histone deacetylases (HDACs), histone methyltransferases (HMTases), and DNA methyltransferases (DNMTs) to target genes [7, 8].

Chimeric fusion oncoproteins in leukemia may also undergo inappropriate forced dimerization [7, 9] which alters the association between the DNA-binding portion in the chimeric oncoproteins and their transcriptional co-factors. This mechanism has been particularly well studied in the core binding factor leukemias, in which the fusion proteins resulting from the chromosomal translocations t(8;21)(q22;q22) (AML1–ETO) and inv(16)(p13q22) (CBFB–MyH11) act as transcriptional repressors [7, 8]. These fusion proteins are characterized by the disruption of CBF, a heterodimeric transcription factor that is important in hematopoietic differentiation. CBF consists of the DNA binding component, AML1 (also known as RUNX1 or CBFA2), and CBFB, which stabilizes AML1. Wild-type AML1 functions as a transcriptional activator. In contrast, in CBF leukemias the dimerization domains of the partners of AML1, which include TEL, ETO, and MTG16, repress the transcription of AML1 target genes by either recruiting HDACs directly or by recruiting co-repressor-HDAC complexes such as N-CoR/Sin3/HDAC1 to AML1 responsive promoters. This keeps the associated histones in the deacetylated state, thereby repressing normal AML1 target genes involved in differentiation and cell cycle inhibition [7–11]. AML1–ETO also directly represses transcription of tumour suppressor genes such as ARF through the AML1 DNA binding domain [12]. HDACs have also been found in complexes with proteins that regulate cell cycle checkpoints such as Rb and its family members [13]. Recently, AML1–ETO has also been shown to recruit DNA methyltransferase 1 (DNMT1) [14] This finding implies that transcriptional silencing of AML1 target genes occurs at least partly through an interplay between histone deacetylation and promoter DNA methylation.

Collectively, these mechanistic insights support the rationale for developing inhibitors of HDAC activity, which, by correcting the transcriptional deregulation of genes involved in cell cycle regulation and apoptosis, could have therapeutic value.

Disruption of HATs in acute leukemia

In addition to the recruitment of HDACs, DNMTs and co-repressor complexes, epigenetic deregulation in acute leukemias and myelodysplastic syndromes also includes a disruption of HATs by gene mutations or chromosomal translocations. Two closely related HATs, E1A-associated 300 kDa protein (p300) and the CREB binding protein (CBP), play distinct but essential roles in hematopoiesis, e.g. as co-activators for several transcription factors including p53, pRB, MYB, JUN, FOS, and RUNX1 [15]. CBP/p300 are regarded as functional tumor suppressors, for mice with inactivated alleles of these gene loci develop hematological malignancies [16].

Cancer-associated mutations abrogating the enzymatic activity of HATs have been observed. CBP/p300 are located on chromosomes 16p13 and 22q13, respectively; regions, which are frequently lost in tumors [17–19]. In addition, rearrangement of both genes has been shown in chromosomal translocations, resulting in aberrant gain or loss of acetylation activity. For example, the t(11;16)(q23;p13) translocation produces mixed lymphcyte leukemia (MLL)-CBP fusion proteins. Unlike wild-type MLL, these fusion proteins cause aberrant acetylation at the HOX loci, which in turn up-regulates the expression of HOX genes and causes the subsequent development of leukemia. A similar mechanism may apply to MLL-p300 fusion proteins derived from the t(11;22)(q23;q13) translocations [11, 15, 20].

The t(8;16)(p11;p13) translocation leads to the production of Monocytic leukaemia zinc finger protein (MOZ)-CBP fusion proteins. MOZ and monocytic leukemia zinc finger protein-related factor (MORF) belong to the MYST family of HATs. In normal hematopoietic cells, MOZ functions as a transcriptional co-activator to potentiate RUNX1-dependent gene expression and stimulate cell differentiation. On contrary, MOZ-CBP fusion proteins down-modulate RUNX1-dependent gene expression and thus lead to leukemogenesis. A similar mechanism may apply to other chromosomal abnormalities involving MOZ and MORF genes, such as inversion of chromosome 8 (inv(8)) which fuses the HAT domain of MOZ to the transcription factor TIF2 (also known as NCOA2). TIF2 is a member of the p160 family of nuclear receptor co-activators known to interact with p300/CBP. MOZ–TIF2 fusion results in the deregulation of transcription through the aberrant recruitment of CBP to nucleosomal regions targeted by MOZ [10, 15].

Epigenetic alterations in acute lymphoblastic leukemia

Transcriptional silencing of genes due to epigenetic mechanisms is an important alteration in acute lymphoblastic leukemia (ALL), with research so far having focused on the role of DNA methylation. An exception is the t(12;21)(p13;q22) (TEL-AML1), which is restricted to precursor B-cell lineage leukemia and is the most common (~25%) translocation in childhood acute lymphoblastic leukemia (ALL). Like AML1 in the CBF acute myeloid leukemias, the abnormal TEL-AML1 fusion protein can bind to core enhancer sequences. Instead of activating transcription through recruitment of co-activators and HATs, it recruits co-repressors and HDACs. Since TEL-AML1-induced transcriptional repression was shown to be reversed by HDAC inhibitors, TEL-AML1-positive ALL may be considered likely to benefit from treatment with HDAC inhibitors [21, 22] Preclinical studies testing this hypothesis are reviewed elsewhere in this paper.

Down-regulation of microRNAs (miRNA) by epigenetic mechanisms, most notably by DNA methylation, may contribute to tumorigenesis. A recent study explores the epigenetic alterations of miRNAs in ALL by analyzing the methylation and chromatin status of the miR-124a loci in ALL [23]. Expression of miR-124a was down-regulated in ALL by hypermethylation of the promoter and histone modifications including decreased levels of acetylated histone H3. Epigenetic down-regulation of miR-124a induced an up-regulation of its target, cyclin-dependent kinase 6 (CDK6) as well as phosphorylation of retinoblastoma (Rb), and contributed to the abnormal proliferation of ALL cells both in vitro and in vivo. CDK6 inhibition by inhibition of HDACs decreased ALL cell growth in vitro. Although an analysis of 353 patients diagnosed with ALL revealed an association between a higher relapse and mortality rate and hypermethylation of the tumor suppressor microRNA Hsa-miR-124a, these results nevertheless provide the rationale for therapeutic strategies in ALL that either target the epigenetic regulation of microRNAs and/or directly target the CDK6-Rb pathway, e.g. by deacetylase inhibitors.

DAC inhibitors in hematologic malignancies: molecular targets and biochemical / pharmacologic properties

At present, 18 HDAC isoforms are known which are grouped into four classes. From a functional as well as from a translational point of view, class I HDACs (isoforms 1,2,3,8) are the best characterized proteins of this family. Less extensive information is available on function and expression of class II isoforms [4–7, 9, 10] while little is known about class III HDACs (the sirtuins) and HDAC11, which on the basis of distinct structural properties has been suggested to constitute an HDAC class on its own.

A large variety of well established as well as novel HDAC inhibitors possess antineoplastic activity *in vitro* and in animal models *in vivo*. Some of these inhibitors are unselective, i.e. they target class I and II but not class III, while others target only specific HDAC classes or isoforms. As these compounds also inhibit deacetylation of numerous non-histone proteins, they should preferably be referred to more broadly as deacetylase inhibitors (DACi). DACi may be subdivided into two categories, which inhibit nuclear and cytoplasmic deacetylases, respectively.

The chemical structure of DACi encompasses three subunits: (1) a zinc-chelating group, (2) a usually hydrophobic “spacer” group, and (3) a catalytic domain which determines the specificity of the compound. DACi inhibitors are classified by structure and include the short-chain fatty acids valproic acid and sodium butyrate, the cyclic tetrapeptides romidepsin (depsipeptide, FK228, FR901228), Trapoxin A, and Apicidin, the hydroxamic acids vorinostat (suberoylanilide hydroxamic acid, SAHA), trichostatin A (TSA), LAQ824, panobinostat (LBH529) and PXD101, the benzamides MS-275, CI-994, und MGCD-0103, cyclic tetrapeptides, electrophilic ketones (trifluoromethylketone), and others (depudecin, SNDX-275, and isothiocyanates) [4]

Although these DACi differ in structure, potency and possibly HDAC enzyme selectivity, they target primarily class I and II HDACs and do not affect the activity of the class III sirtuins (reviewed by Marchion and Münster [24]) It is becoming increasingly clear that the downstream effects of HDAC inhibition, which ultimately leads to growth inhibition and apoptosis in different tumor types, depend upon both the HDAC inhibitor and the cell type [25–27]. Only very recently, research began to focus on the histone acetylation status in human tumours in general and the specific expression of HDAC isoforms in solid as well as hematological malignancies.

Non-histone targets of DACi in acute myeloid leukemia

HATs and DACs affect the acetylation status of lysine residues not only of histones but also of transcription factors (TF) (eg, p53, E2F1, GATA1, RelA, Y1, MAD/MAX, TFIIE and TFIIF, and hormone receptors). Altered acetylation of TFs may affect their DNA binding and transcriptional activity [4–6, 28]. In addition, DACs have been shown to deacetylate a multitude of proteins other than histones or transcription factors, e.g. the cytoskeleton protein-tubulin, β-catenin, DNA repair enzymes and the heat shock protein 90 (hsp90) [29–35]. Accordingly, modulation of DAC function by DACi may not only affect gene transcription but also modify the stability of

proteins, as well as the ability of proteins to interact with DNA and other proteins involved in important biologic functions in the leukemic cells [36]. The sheer number of transcription factors known to be acetylated suggests that the acetylation of these nonhistone proteins may have as much regulatory effect on transcription as the acetylation of histone proteins. A more comprehensive description of nonhistone targets of acetylation has been reviewed recently [36–38].

The molecular basis for the relatively selective antitumor activity of DACi is unknown. Insigna et al. investigated the effects of DACis on leukemias expressing PML-RAR or AML1-ETO [39]. Even though these oncoproteins are known to initiate leukemogenesis through deregulation of HDACs, it was shown that oncogene expression is not sufficient to confer DACi sensitivity to normal cells. DACi-induced induction of apoptosis in leukemic cells was found to be p53 independent and depend upon activation of the death receptor pathway (TRAIL and Fas signaling pathways). Interestingly, TRAIL, DR5, FasL and Fas were upregulated by DACis in the leukemic cells, but not in normal hematopoietic progenitors, indicating that sensitivity in leukemias is a property of the fully transformed phenotype and depends on DACis-induced activation of a specific death pathway.

DACi-mediated induction of TRAIL was also identified as a mediator of the selective anticancer action by Nebbioso et al. [40]. Expression of TRAIL, by directly activating the TNFSF10 promoter, triggered tumor-selective death signaling in acute myeloid leukemia (AML) cells, without inducing apoptosis in normal CD34(+) progenitor cells. DACi induced proliferation arrest, TRAIL-mediated apoptosis and suppression of AML blast clonogenicity occurred irrespective of karyotype.

Heat shock proteins (HSPs) are molecular chaperones that stabilize folding and conformation of both normal and oncogenic proteins. These chaperones thereby prevent the formation of protein aggregates. HSPs are often overexpressed in human malignancies, including AML (see [41] for review), and are the main chaperones required for the stabilization of multiple oncogenic kinases involved in the development of AML. HSP90 client proteins are involved in the regulation of apoptosis, proliferation, autophagy and cell cycle progression; several of these proteins are considered possible therapeutic targets for treatment of AML. The results from initial phase I/II clinical trials testing HSP90 inhibitors have documented that HSP90 inhibition can mediate antileukemic effects in vivo. HSP90 activity is also regulated by posttranscriptional modulation, and HSP90 inhibition can thereby be indirectly achieved through increased acetylation caused by DACi (see section Combination with other agents)

Preclinical studies of DAC inhibitors in acute leukemias

Single-agent DACi in AML

Valproic acid (VPA) has a long history of use as an antiepileptic drug. After *in vitro* studies demonstrated that VPA induced differentiation of carcinoma cells and leukemic blasts derived from patients with AML, it was discovered that VPA acts as a DACi [42]. In addition, VPA causes selective proteasomal degradation of HDAC2, but not of other class I HDACs (i.e., HDAC 1, 3, and 8) [42].

In t(8;21) acute myeloid leukemia (AML), the AML1/ETO fusion protein promotes leukemogenesis by recruiting class I HDAC-containing repressor complex to the promoter of AML1 target genes, as described above. VPA disrupts the physical interaction between AML1/ETO and HDAC1, stimulates the global dissociation of the AML1/ETO-HDAC1 complex from the promoter of AML1/ETO target genes, and induces relocation of both the AML1/ETO and HDAC1 proteins from the nucleus to a perinuclear region. Mechanistically, these effects are associated with a significant inhibition of HDAC activity, histone H3 and H4 hyperacetylation, and recruitment of RNA polymerase II, resulting in transcriptional reactivation of target genes (i.e., IL-3) otherwise silenced by the AML1/ETO fusion protein. Ultimately, these pharmacological effects resulted in significant antileukemic activity mediated by partial cell differentiation and caspase-dependent apoptosis [43].

VPA was recently shown to enhance proliferation and self-renewal of normal hematopoietic stem cells, raising the possibility that VPA may also support growth of leukemic progenitor cells (LPC). Indeed, VPA maintained a significantly higher proportion of CD34(+) LPC and colony forming units compared to control cultures in six AML samples, but selectively reduced leukemic cell numbers in another AML sample with expression of AML1/ETO. These data suggest a differential effect of VPA on the small population of AML progenitor cells and the bulk of aberrantly differentiated blasts in the majority of AML samples tested [44].

The new hydroxamic acid derivative, ITF2357, blocked proliferation and induced apoptosis in AML1/ETO-positive Kasumi-1 and primary blast cells in concentration of 0.1 μM, whereas AML1/ETO-negative HL60, THP1 and NB4 cell lines were sensitive only to 1 μM ITF2357. In Kasumi-1 cells, ITF2357 induced AML1/ETO degradation through a caspase-dependent mechanism and also determined DNMT1 efflux from, and p300 influx to, the nucleus. Moreover, ITF2357 induced local H4 acetylation and release of DNMT1, HDAC1 and AML1/ETO, paralleled by recruitment of p300 to the IL-3 gene promoter. ITF2357 treatment, however, did not induce re-expression of IL-3 gene. Accordingly, the methylation level of IL-3

promoter, as well as of several other genes, was unmodified [45].

As VPA and ITF2357 seem to specifically target AML1/ETO-driven leukemogenesis, integration of both class I-selective and pan-DACi in novel therapeutic approaches for AML1/ETO-positive AML may be worthwhile.

Single-agent DACi in acute lymphoblastic leukemia

The potential anti-leukemic activity of DACi in ALL has been examined in a small number of preclinical studies; most of these tested DACi as a single agent *in vitro* using human ALL cell lines as models.

An early study compared the *in vitro* effects of the cyclic tetrapeptide romidepsin (FK228) on human leukemia/lymphoma cells and cell lines with normal hematopoietic cells [46]. Romidepsin induced G1 arrest and / or apoptotic cell death at nanomolar concentrations. Clinical samples from patients with ALL were more sensitive to romidepsin at clinically achievable drug concentrations than either normal peripheral blood or bone marrow mononuclear cells or normal progenitor cells. Expression levels of HDAC-1 and HDAC-3 proteins did not correlate with the sensitivity to romidepsin.

The anti-leukemic activity and mode of action of the hydroxamic acid derivative, LAQ824 was studied using four human pre-B lymphoblastic cell lines as models representing different cytogenetic subsets (Sup-B15 and TMD-5, both t(9;22) positive, SEM, t(4;11) positive, and NALM-6 cells). LAQ824 significantly inhibited the proliferation of leukemic lymphoblastic cell lines; this was due to increased apoptosis accompanied by activation of caspase-3 and caspase-9, cleavage of poly(ADP-ribose)-polymerase (PARP) as well as by down-regulation of Bcl-2 and disruption of the mitochondrial membrane potential. Surprisingly, LAQ824-induced apoptosis was partially independent of caspase activation [47].

Panobinostat (LBH589), a broad-spectrum DACi closely related to the hydroxamate LAQ824 but with more favorable pharmacologic properties, potently induced cell-cycle arrest, apoptosis, and histone (H3K9 and H4K8) hyperacetylation in two human cell line models of Philadelphia chromosome-negative acute lymphoblastic leukemia (T-cell MOLT-4 and pre-B-cell Reh). Panobinostat treatment increased mRNA levels of proapoptosis-, growth arrest-, and DNA damage repair genes including FANCG, FOXO3A, GADD45A, and GADD45B. This was associated with increased histone acetylation at the GADD45G promoter and phosphorylation of histone H2AX. Treatment with panobinostat also induced apoptosis in primary ALL cells, including those from a relapsed patient [48].

A comparison of the growth inhibitory and apoptotic activity of several DACi's that differ in their subclass selectivity towards glucocorticoid sensitive and resistant acute lymphoblastic leukemia cells (ALL) and patient blasts was reported by Tsapis et al. [49]. Vorinostat (SAHA) displayed similar efficacy on glucocorticoid-sensitive and insensitive ALL cells but did not synergize with dexamethasone. Both B-precursor and T-ALL cells were much more efficiently killed by VPA and by the pan-DACi vorinostat than by the sub-class I selective DAC inhibitor MS275. This group´s previous observation that MS275 and vorinostat displayed a similar growth inhibitory and proapoptotic activity in the AML cell line U937 suggest that cellular context may be an important determinant of the biologic response to DACi. The mechanisms of vorinostat-induced apoptosis in ALL likewise were cell-line dependent, acting through initiator caspase 10 in CEM-C7 cells while inducing apoptosis through the intrinsic, as well as through caspase-independent death pathways in CEM-C1 cells. In addition, vorinostat induced re-expression of DR5 in glucocorticoid-insensitive CEM-C1 cells that do not express DR5 and are insensitive to TRAIL, indicating that vorinostat may overcome glucocorticoid resistance by inducing alternative death pathways. This ability of DACi to induce apoptosis in glucocorticoid-resistant ALL was confirmed in a more recent report [49]. Taken together, these studies establish that several DACi possess potent growth inhibitory activity against B-precursor and T-cell ALL largely independent of specific cytogenetic aberrations such as TEL-AML1 [50]. Anti-leukemic activity is associated with up-regulation of genes critical for DNA damage response and growth arrest. These findings are not restricted to the in vitro setting, as DACi have demonstrated potent antitumor activity in two NOD/SCID mouse models of B-cell precursor childhood ALL [51].

Combination of DACi with differentiation-inducing agents in AML and MDS

Treatment with DACi's alone or in combination with either all-trans retinoic acid (ATRA) or granulocyte colony-stimulating factor is able to overcome inhibition of differentiation caused by chimeric fusion oncoproteins such as PMLRARα, PLZF-RARα, or AML-ETO [5, 52–55]. In addition, combination of DACi's with ATRA induces differentiation of APL cells that are resistant to treatment with ATRA alone [54]. Recently, primary leukemia blasts were shown to undergo differentiation following treatment with a combination of ATRA and DACi's [42, 55].

Combination with hypomethylating agents

Functionally, histone acetylation and DNA methylation are tightly linked. For example, DNA methyltransferases

(DNMTs) direct different HDACs to specific sections of chromatin B-precursor and T-cell with repressed genes and thereby augment gene repression. Since DNA methylation is the principal mechanism for suppressing gene expression, methylated genes are typically resistant to treatment with DACi alone. However, sequential exposure of malignant cells with a DNMT-inhibitor followed by a DACi had synergistic effects on reactivation of gene expression and induction of apoptosis [56, 57].

The anti-apoptotic transcription factor NFkappaB is constitutively activated in myeloblasts arising in high-risk myelodysplastic syndromes (MDS) and AML. Recent studies indicate that DNMT inhibitors (such as azacytidine and decitabine) and DACi (such as trichostatin A and VPA) can inhibit the constitutive activation of NFkappaB in malignant myeloblasts *in vitro* and *in vivo*. The rapid onset of this effect within a few hours suggested that it was not due to epigenetic reprogramming; indeed, DNMT and HDAC inhibitors reduced the phosphorylation of the NFkappaB-activating kinase IKKα/ß. Thus, inhibition of NFkappaB and IKKα/ß by DNMT inhibitors in concert with DACi may contribute to the antileukemic activity of these classes of agents [58].

Combined treatment with panobinostat and decitabine may potentiate antileukemia activity against human AML cells by other mechanisms as well. The histone methyltransferase EZH2 modulates DNA methylation by recruiting DNMT1 to the DNA. Exposure to the pan-DACi panobinostat reduces DNMT1 and EZH2 protein levels by inhibiting the chaperone association of Hsp90 with DNMT1 and EZH2. This promotes their proteasomal degradation and disrupts the interaction of DNMT1 with EZH2. In K562 myeloid leukemia cells, co-treatment with panobinostat and decitabine caused greater diminution of DNMT1 and EZH2 levels than either agent alone. Exposure to panobinostat and decitabine in combination augmented induction of apoptosis of primary AML but not normal CD34(+) bone marrow progenitor cells and may thus exerts clinically relevant antileukemia activity against human AML cells [59].

Combination of DACi with chemotherapy

Molecular perturbations resulting from exposure to DACi lower the threshold for apoptosis by multiple mechanisms. This supports the rationale for combining DACi with conventional cytotoxic agents, which generally trigger apoptosis by perturbing cell cycle and/or inducing DNA damage [28]. Additional support for this basic concept stems from studies in solid tumors, demonstrating that co-treatment with DACi, e.g. vorinostat or LAQ824, significantly enhances the cytotoxic effects of docetaxel, epothilone B, and gemcitabine against human breast cancer cells [34], of fluorouracil against colorectal cancer and of etoposide, camptothecin, doxorubicin, and cisplatinum against glioblastoma cells [60, 61]. Co-treatment with phenylbutyrate has also been documented to increase the activity of conventional chemotherapeutic agents commonly used in treatment of acute leukemias such as cytarabine and etoposide, against non-Hodgkin's lymphoma, chronic lymphoid leukemia (CLL), and multiple myeloma cells [62, 63].

The potential value of combining DACi with topoisomerase II-inhibitors is based on preclinical studies suggesting a DACi-mediated stimulation of the enzyme in human leukemic cells [64, 65]. Anthracyclines induce DNA double strand breaks by forming a complex with both the DNA and the DNA repair enzyme topoisomerase II, which disrupts DNA replication and transcription [66].

The pan-DACi panobinostat was tested in combination with doxorubicin on AML cell lines and primary leukemia blasts and induced cell death by an increase in the mitochondrial outer membrane permeability and release of cytochrome c from the mitochondria, resulting in caspase-dependent apoptosis accompanied by upregulation of Bax, Bak and Bad. The drug combination provoked a strong activation of a DNA damage response, indicating that this combination may trigger cell death by a mechanism that induces DNA double-strand breaks [67]. These data are consistent with a report by Sanchez et al, which showed that simultaneous exposure of AML cells to idarubicin and VPA or vorinostat *in vitro* led to a significant and synergistic increase in the proportion of apoptotic cells [66]. Taken together, these data indicate that the combination of panobinostat and an anthracycline may be an effective therapy for the treatment of AML.

Apart from anthracyclines, cytosine arabinoside (ara-C) and etoposide are the most widely used chemotherapeutic agents for treatment of AML. The DACi vorinostat was shown to induce topoisomerase II expression and sensitize human leukemia cells to etoposide and other inhibitors of topoisomerase II [65]. The combination of etoposide or ara-C with vorinostat was evaluated in a preclinical study using cell proliferation assays and cell cycle analysis to determine the effects on HL-60 myeloid leukemia cells and K562 myeloid blast crisis chronic myelogenous leukemia cells. The sequence of drugs proved to be important: cytotoxic antagonism resulted when vorinostat was combined concomitantly with ara-C, whereas the sequential administration with vorinostat given prior a drug-free interval followed by ara-C was mostly synergistic. Etoposide combined with vorinostat was additive to synergistic, and the synergism became more pronounced when etoposide was given after vorinostat. Cell cycle analyses revealed that the sequence-dependent interaction of vorinostat and ara-C or etoposide reflected the arrest of cells in G1 or G2 phase

during vorinostat treatment and recovery into S phase after removal of vorinostat [68].

Interactions between the novel benzamide DACi MS-275 and fludarabine were examined in lymphoid and myeloid leukemia cells in relation to mitochondrial injury, signal transduction events, and apoptosis. Prior exposure of Jurkat lymphoblastic leukemia cells to a marginally toxic concentration of MS-275 for 24 hours sharply increased mitochondrial injury, caspase activation, and apoptosis in response to a minimally toxic concentration of fludarabine, resulting in highly synergistic antileukemic interactions and loss of clonogenic survival. Simultaneous exposure to MS-275 and fludarabine also led to synergistic effects, but these were not as pronounced as observed with sequential treatment. Similar interactions were noted with myeloid cell lines, other DACi (e.g., sodium butyrate) and other nucleoside analogues (gemcitabine). Potentiation of fludarabine lethality by MS-275 was associated with acetylation of histones H3 and H4, down-regulation of the antiapoptotic proteins XIAP and Mcl-1, enhanced cytosolic release of proapoptotic mitochondrial proteins (e.g., cytochrome c, Smac/DIABLO, and apoptosis-inducing factor), and caspase activation. It was also accompanied by the caspase-dependent down-regulation of p27, cyclins A, E, and D1, and diminished phosphorylation of retinoblastoma protein. Combined exposure of cells to MS-275 and fludarabine was associated with a significant increase in generation of reactive oxygen species (ROS); moreover, both the increase in ROS and apoptosis were largely attenuated by coadministration of the free radical scavenger L-N-acetylcysteine [69].

The drugs used in ALL-specific regimens differ considerably from those employed in treatment of AML. The antifolate methotrexate (MTX), a universal component of ALL therapies, is metabolized by folylpoly-gamma-glutamate synthetase (FPGS) into long-chain polyglutamates (MTX-PG(3-7)), resulting in enhanced cytotoxicity from prolonged inhibition of dihydrofolate reductase (DHFR) and thymidylate synthetase (TS). Identification of a hypersensitive site upstream from exon-1 of the FPGS gene suggested that chromatin remodeling could alter FPGS expression. Leclerc et al. demonstrated that HDAC1 is recruited by NFY and Sp1 transcription factors to the FPGS promoter in ALL cell lines. Exposure of these cell lines to the DACi´s sodium butyrate and vorinostat increased FPGS mRNA expression by 2- to 5-fold, whereas DHFR and TS mRNA expression was decreased. Combination treatment with MTX plus vorinostat significantly enhanced cytotoxicity and apoptosis in B- and T-ALL cell lines as compared with each drug alone. Vorinostat lead to the intracellular accumulation of long-chain MTX-PG(3-7). Therefore, DACi-induced FPGS expression increases the accumulation of MTX-PG(3-7) and cytotoxicity in ALL cell lines, which is potentiated by DHFR and TS downregulation [70].

Taken together, the findings indicate that DACi induces multiple perturbations in signal transduction, survival, and cell cycle regulatory pathways that lower the threshold for chemotherapy-mediated mitochondrial injury and apoptosis in human myeloid and lymphoid leukemia cells. These data also provide insights into possible mechanisms by which novel, clinically relevant DACi might be used to enhance the antileukemic activity of a wide spectrum of standard chemotherapeutic agents, including anthracyclines, nucleoside analogues such as fludarabine, topoisomerase inhibitors and antimetabolites. While these observations suggest that the combination of DACi and cytotoxic drugs may enhance the efficacy of therapy for AML and ALL, there is evidence that the sequence of drug administration may be of paramount importance, and that inattention to proper scheduling of these agents may even be antagonistic. Furthermore, the effect of combination treatment on non-hematologic organ toxicity is unknown. Other caveats include the possibility of drug-drug interactions and the theoretic potential of DACi´s to induce drug resistance by a variety of mechanisms, as will be discussed below (see section "DACi—associated drug resistance"). Ultimately, well-controlled phase I and II studies will be necessary to determine the feasibility and clinical utility of these promising strategies.

Combination with other agents

Chimeric oncoproteins with constitutive tyrosine kinase activity such as BCR-ABL and mutant receptor tyrosine kinases such as mutant fms-like tyrosine kinase 3 (FLT3) contribute to the leukemic phenotype in a variety of acute and chronic leukemias and are therefore targeted clinically by selective inhibitors of the respective tyrosine kinases. In addition, these aberrant kinases are client proteins of the molecular chaperone heat shock protein 90, raising the possibility that inhibition of HSP90 could lead to depletion of these oncogenic proteins and act synergistically with TKIs in mediating anti-leukemic effects. The pan-DACi LAQ824 induces acetylation and inhibition of HSP90. When biphenotypic leukemia MV4-11 cells were treated with LAQ824, levels of FLT-3 and p-FLT-3 were attenuated due to enhanced proteasomal degradation of these kinases. Cotreatment with LAQ824 and PKC412 synergistically induced apoptosis of MV4-11 cells and enhanced apoptosis of primary AML cells expressing mutant FLT-3. These data support the notion that the combination of LAQ824 and PKC412 is highly active against human AML cells with mutant FLT-3 [71].

The effect of the DACi MS-275 against a variety of human leukemia cells with defined genetic alterations was

explored by Nishioka et al. [72]. MS-275 induced growth arrest of MOLM13 and MV4-11 AML cells, which possess internal tandem duplication mutation in the FLT3 gene (FLT3-ITD). Exposure of these cells to MS-275 decreased levels of total, as well as, phosphorylated forms of FLT3, resulting in inactivation of its downstream signal pathways, including Akt, ERK, and STAT5. Further studies found that MS-275 induced acetylation of Hsp90 in conjunction with ubiquitination of FLT3, leading to degradation of FLT3 proteins in these cells. The action of MS-275 in leukemia cells was potentiated by inhibition of MEK/ERK signaling, suggesting that DAC inhibition may be useful for treatment of individuals with leukemia possessing activating mutation of FLT3 gene.

An analogous experimental approach was taken in a study investigating the effect of MS-275 in combination with pharmacological blockage of Akt/mammalian target of rapamycin (mTOR) signaling in HL60 AML and NB4 acute promyelocytic leukemia (APL) cells [72]. Inactivation of mTOR by the rapamycin analog RAD001 (everolimus) significantly enhanced MS-275-mediated growth inhibition and apoptosis of these cells. When used in combination, RAD001 potentiated the ability of MS-275 to induce differentiation of HL60 and NB4 cells. Proliferation of HL60 tumor xenografts in nude mice was significantly inhibited by combining RAD001 with MS-275, without adverse effects. Taken together, concomitant administration of a DACi and an mTOR inhibitor may be a promising treatment strategy for the individuals with a subset of human leukemia.

Although VPA is a less potent DACi than the hydroxamic acid or benzamide derivatives, strong anti-proliferative and pro-apoptotic effects of VPA were observed on human ALL and CML cell lines at concentrations achievable in vivo. These effects were most pronounced in ALL cell lines as well as in primary ALL cells. Notably, VPA revealed enhanced activity with imatinib mesylate, nilotinib, the farnesyl transferase inhibitor SCH66336, interferon-alpha and cytosine arabinoside. VPA inhibited the growth of colony-forming cells from 12 Ph + chronic-phase CML patients, but also of those from normal healthy controls in a dose-dependent fashion. In conclusion, VPA, whether alone or in combination with other non-classical anti-leukemic compounds, exerts significant anti-leukemic effects on human ALL and CML cells [73].

The proteasome has been successfully targeted for the treatment of multiple myeloma and mantle cell lymphoma, but proteasome inhibition alone has been clinically less effective in other hematologic malignancies. In leukemic cell lines, the novel proteasome inhibitor NPI-0052 and DACi synergistically induced apoptosis in leukemia cells in a caspase-8- and oxidant-dependent manner. In leukemic mice, treatment with NPI-0052 reduced the white blood cell (WBC) burden over 35 days. The combination of NPI-0052 with the DACi MS-275 or valproic acid was more effective than the combination of bortezomib with these DACi's [74]. Further studies aimed at identifying mechanisms of synergy revealed that NPI-0052 elicits caspase-8 and oxidative stress-dependent epigenetic alterations, and that DAC inhibition repressed expression of the proteasomal beta5, beta2, and beta1 subunits, consequently inhibiting respective enzymatic activities. These results indicate that crosstalk between NPI-0052 and DACi contributes to the synergistic cytotoxic effect on leukemia cells. This reinforces the potential clinical utility of combination studies of DACi and proteasome inhibitors [75].

Clinical experience with deacetylase inhibitors in MDS and AML

To date, clinical examination of DACi in patients with AML has met with limited success. Current experience is mostly restricted to phase I or phase II studies involving small numbers of patients.

Valproic acid is the only DACi that has been clinically investigated in larger numbers of patients with MDS. An early clinical trial involving 18 MDS patients showed an overall response rate of 37% (1 partial response (PR) and 6 hematologic improvements (HI)). All-*trans* retinoic acid (ATRA) did not enhance the clinical activity in patients who had not already demonstrated a response to VPA [76]. In a subsequent report of 43 patients with MDS, the overall response rate was 35% (1 PR, and 15 HI) [77]. Efficacy of the VPA +/- ATRA treatment seems to be inversely correlated with the stage of the disease. According to Kuendgen et al., low or intermediate-I IPSS scores or a normal bone marrow blast count proved to be good predictors for response [78].

The combination of VPA with ATRA was also tested in AML patients who were considered ineligible for intensive chemotherapy. A disappointingly low response rate of only 5% with no CR was observed among 40 patients receiving this combination [79] Similarly, none of 26 patients with high-risk AML achieved a CR in another study examining combined treatment with VPA and ATRA [80]. Results did not improve when ATRA was added later on in another pilot study with 8 AML patients [81]. Nevertheless, 5 out of 11 patients with de novo AML responded to a therapy with VPA, ATRA and theophylline (1 CR, 2 complete remissions with incomplete recovery of peripheral blood counts (CRi), 2 HI) [82].

In view of the development of the novel, considerably more potent DAC inhibitors vorinostat, panobinostat, romidepsin and the isotype-specific MGCD0103, the use of VPA in patients with AML, as a single agent or in

combination with ATRA, does not appear very promising. The role of VPA in combination with hypomethylating agents will be addressed in a later section of this review.

Vorinostat was investigated as a single agent in a phase I study with 31 AML and 10 MDS patients. The maximal tolerated dose was 200 mg BID. Seven patients experienced hematologic improvement, including 4 CR in patients with AML. Increased histone acetylation was observed at all dose levels [83]. These favorable results were not confirmed in a randomized phase II trial, in which only one out of 37 AML patients achieved CR [84].

Panobinostat (LBH589) was administered in a phase I study as a 30-min infusion on days 1 to 7 of a 21-day cycle. Fifteen patients with AML (n=13), ALL (n=1), or MDS (n=1) were treated. At doses <11.5 mg/m^2, i.v. panobinostat was well tolerated with consistent, albeit transient antileukemic and biologic effects: in 8 of 11 patients peripheral blasts declined, but rebounded following the 7-day treatment period. The median acetylation of histones H2B and H3 in CD34(+) and CD19(+) cells increased significantly during therapy, as did apoptosis in CD14(+) cells [85]. Oral panobinostat was evaluated in patients with advanced hematologic malignancies. Doses of ≥ 40 mg weekly lead to 2 CRs out of 26 evaluable AML patients [86].

Romidepsin is a potent, bicyclic tetrapeptide with DAC-inhibitory activity. Twenty patients with AML were treated at 13 mg/m(2)/d on days 1, 8, and 15 of a 28-day cycle. Antileukemic activity was observed in 5 of 7 patients with chromosomal abnormalities known to recruit HDACs, including those involving core binding factor (CBF). Two patients had clearance of bone marrow blasts and 3 patients had a greater than 50% decrease in bone marrow blasts. These responses were associated with a significant increase in MDR1, p15 and p14 expression [87]. In a phase II study in patients with high-risk MDS and AML, one patient achieved a CR, 6 patients experienced stable disease [88].

The hydroxamate DACi belinostat was administered as a 30-min i.v. infusion on days 1-5 of a 21-d cycle to 16 patients with advanced hematologic malignancies. No complete or partial remissions were noted in these heavily pre-treated patients [89].

In contrast to the potent pan-DACi panobinstat, belinostat and vorinostat, the orally administered benzamide MGCD0103 is a selective inhibitor of HDAC1, 2, 3 (class 1) and 11 (class 4) and does not inhibit class 2 HDACs. In a phase I study, 3 of 29 patients with leukemia or MDS achieved a CR [90].

A phase I dose escalation study of the synthetic benzamide derivative MS-275 was completed in 75 patients with advanced acute leukemias. In spite of biologic alterations such as increase in protein and histone H3/H4 acetylation, p21 expression, and caspase-3 activation in bone marrow mononuclear cells, no clinical responses were seen [91].

Toxicity of deacetylase inhibitors

The most frequently adverse effects observed during DACi treatment for hematologic malignancies include gastrointestinal toxicity with nausea, vomiting and diarrhea, as well as fatigue, thrombocytopenia, neutropenia and non-specific ECG changes such as flattened T-waves, ST-segment depression, and QT-prolongation [92].

These changes are dose-dependent and represent a class effect of DACi. The pathogenesis of thrombocytopenia is unclear, but does not appear to involve cytotoxic mechanisms, as they are reversible within a few days of drug discontinuation. The other adverse events typically also rapidly revert to normal, or are easily controlled by prompt institution of supportive therapy, e.g. in the case of diarrhea. Nevertheless, the different DACi display some notable differences regarding their toxicity profiles.

Epigenetic therapy combining deacetylase- and DNA-methyltransferase-inhibitors and/or ATRA in AML and MDS

The first clinical studies to combine DACi and hypomethylating agents in patients with MDS and AML were conducted with VPA (see Table 1). Promising results were obtained in each of these studies [93–96] indicating both improved and accelerated responses with combined epigenetic therapy: the time to response was significantly shorter with combined therapy (1–3 cycles) than with single-agent DNMT (4–6 cycles). VPA was targeted to therapeutic plasma levels of at least 50 μg/mL to increase the efficacy of the hypomethylating agent [96], but no association between histone acetylation and response was observed [97]. Further correlative studies demonstrated reversal of p15 or CDH-1 promoter methylation during the first cycle of therapy in all six responding patients, whereas no demethylation was observed in any of the six non-responders [97].

Even though these trials were designed primarily as dose-finding studies, the 20–30% remission rate (CR and CRi) was higher than generally observed with 5-azacitidine or decitabine when used as a single agent. This also holds true for a combination trial of SAHA and decitabine: of 61 patients evaluable for response, a CR or CRi was achieved by 18% patients with MDS, 8% with relapsed/refractory AML, and 36% with untreated AML. [98] Even higher CR/CRi rates of up to 40 to 60% were achieved in untreated patients with AML by combining a hypomethylating agent

Table 1 Clinical trials evaluating DACi in combination with DNMT inhibitors

	VPA (d 1–10) + Decitabine (d 1–10) [93],	VPA (d 5–21) + Decitabine (d 1–10) [94]	VPA + Azacitidine (d 1–7) + ATRA (d 3–7) [95]	VPA cont. + Azaciditine (d 1–7) [96]	Phenylbutyrate (7 d cont.) + Azacitidine (d1–14 max.) [97]
Patient numbers	*N*=54	*N*=25	*N*=53	*N*=62	*N*=32
Diagnosis	AML (89%), MDS (11%)	AML	AML (92%) MDS (8%)	MDS	AML (56%) MDS (44%)
Response (CR, CRi)	22%	32%	28%	12%	9%
No of previously untreated patients	11	12	33	na	8
Response of previously untreated patients	50%	58%	42%	na	25%
Survival of responding patients	median 15 months	3–10 months	> 5 months	na	8–19+ months

with VPA. A recent follow up of these untreated patients revealed that responders received more cycles of therapy and had significantly longer survival. Non-responding patients had a higher WBC and higher bone marrow blast count at the start of therapy. In general, patients who relapse after combination epigenetic therapy appeared to have a poor prognosis [99].

Numerous further combination studies using potent HDACi are currently being conducted, the results of which are being awaited with interest.

Combination of deacetylase inhibitors with cytotoxic agents

The rationale for phase I trials of the sequential combination of vorinostat followed by cytotoxic agents in patients with acute leukemias stems from *in vitro* studies showing that the sequence-dependent interaction of vorinostat and cytarabine or etoposide arrested the cells in G1 or G2 phase during vorinostat treatment and allowed recovery into S phase after removal of vorinostat [68]. Concurrent administration of vorinostat and idarubicin for 3 days was demonstrated in a phase I trial in 41 patients with refractory leukaemia (90% AML), with 2 CRs, 1 CRi and 4 marrow responses. Correlative studies demonstrated histone acetylation in patients on therapy and modulation of CDKN1A and TOP2A (topoisomerase II) gene expression and a dose-related elevation in plasma vorinostat concentrations [100].

Preliminary results of a phase II study of vorinostat followed by idarubicin and cytarabine with 45 mostly high-risk AML patients aged > 65 years were reported. Induction therapy consisted of oral vorinostat 500 mg TID (days 1 to 3), idarubicin 12 mg/m^2 iv (days 4 to 6) and cytarabine (1.5 g/m^2 as a continuous infusion on days 4 to 7) followed by a maximum of 5 consolidation cycles with dose-reduced chemotherapy. Complete remission (CR) after one course of therapy was achieved in 35 patients and 1 patient achieved a CRi for an overall response rate of 80%. No excess toxicity with the addition of vorinostat has been observed compared to standard induction therapy [101].

DACi-associated drug resistance

The rationale for developing deacetylase inhibitors as anti-leukemic agents is based on evidence showing induction of cellular differentiation, growth arrest, and apoptosis of malignant cells, but there is no evidence that only genes involved in leukemogenesis are targeted by DACi. Recent reports indicate that expression of the multidrug resistance-1 (MDR1) gene is also regulated by epigenetic mechanisms, raising the possibility that this and other drug transporters able to counteract the cytotoxicity of various anti-leukemic drugs may be upregulated by DACi. This was in fact shown to be the case in a recent *in vitro* study demonstrating that in AML cells, expression of MDR1, breast cancer resistance protein (BCRP), and multidrug resistance-associated proteins (MRP) 7 and 8 were induced by phenylbutyrate, valproate, vorinostat or trichostatin A in a dose- and time-dependent manner. The pattern of gene induction by different DACi was shown to be cell line specific and associated with hyperacetylation of histone proteins in the promoter regions of MDR1, BCRP, and MRP8. Drug-induced apoptosis was impaired in KG-1α cells treated with phenylbutyrate, resulting in resistance to daunorubicin, mitoxantrone, etoposide, vinblastine, paclitaxel, topotecan, gemcitabine, and 5-fluorouracil [102].

Similarly, the combination of ATRA with depsipeptide (FK228) induced MDR1 expression in NB4 promyelocytic leukemia (APL) cells, which normally do not express MDR1 and are highly sensitive to anthracyclines. Upregulation of MDR1 expression occurred via increased H4 and

H3-Lys9 acetylation of the MDR1 promoter, and prevented doxorubicin-induced growth inhibition and apoptosis in APL cells. G1 cell-cycle arrest and upregulation of p21 mRNA may have further impaired the induction of apoptosis of cells in G2 phase. Conversely, initial exposure to doxorubicin followed by ATRA/FK228 treatment enhanced apoptosis [103]. These results indicate that epigenetic mechanisms leading to a drug resistance phenotype broader than the "classic multidrug resistance" may be activated by DACi exposure of AML cells, an effect which might impair therapeutic efficacy. This highlights the importance of investigating mechanism-based sequential therapies in clinical trials that combine DAC inhibitors with other agents commonly used for treatment of acute leukemias.

DACi as treatment for acute leukemias and MDS: a critical appraisal and future perspectives

Despite the profound preclinical activity of DACi against acute myeloid leukemia cells, their clinical development as treatment for myeloid malignancies has been less straightforward than for several lymphoid malignancies, most notably cutaneous T cell lymphoma. The to date modest success in AML and MDS highlights the limitations of our understanding of the complex interactions between epigenetic and genetic changes in these malignancies, and the paucity of our knowledge concerning the biological function of individual HDAC enzymes and the pleiotropic cellular effects of DACi. While it has been established that treatment of leukemic cells with DACi induces cell death, differentiation and/or cell-cycle arrest, they may also effect neoplastic growth by influencing the tumor microenvironment, regulating host immune responses or modifying the properties of normal hematopoietic cells. Thus, the beneficial effect of valproic acid in patients with low risk MDS may actually be a reflection of the unique ability of VPA to enhance normal hematopoietic function, whereas patients with high risk MDS or AML generally do not benefit because of the insufficient anti-leukemic activity of VPA in conjunction with the depletion of normal, potentially VPA-responsive progenitor cells.

In addition, the initial concept that DACi mediate their biological effects only through the regulation of gene expression via direct hyperacetylation of histones is no longer valid, following realisation that DACi acetylate diverse non-histone proteins, thereby regulating a broad range of cellular functions independent of transcriptional mechanisms. It is therefore not surprising that the actions of DACi are cell-context dependent, and will differ according to the differential expression and function of the individual HDACs in a given leukemia. HDAC enzyme expression is therefore likely to determine differential sensitivity to various types and dosages of DAC inhibitors, as well as their toxicity profile.

Limited clinical experience has so far demonstrated the relative safety of DACi currently in clinical testing, but adverse effects that become particularly relevant with long-term use have been noted, some of which, such as fatigue and diverse gastrointestinal complaints, appear to be class effects. Moreover, safety aspects will need to be reevaluated when DACi are combined with other anticancer agents, an obvious next step in their clinical development given available evidence that the clinical benefit derived from epigenetic and chromatin modifiers will be accrued when they are combined with chemotherapy or other targeted anti-leukemic agents. The potential of DACi to upregulate drug efflux pumps is a notable example for potentially detrimental drug-drug interactions, although there is as yet no evidence that this is clinically relevant. A rational selection of appropriate combination partners for DAC inhibitors will require an improved understanding of the specific epigenetic and genetic aberrations of each hematologic malignancy, which will have to be complemented by the identification of predictive biomarkers. If these challenges can be met, DAC inhibitors should develop into an important element of novel, targeted treatment strategies for leukemia.

Conflict of Interest Gesine Bug: Honoraria from Novartis for Advisory Boards and presentations; Celgene for presentations.

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